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4TU metabolic labeling and RNA analysis

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An abbreviated version of this protocol was published in eLIFE in Sep 2018

Non-invasive measurement of mRNA decay reveals translation initiation as the major determinant of mRNA stability

DOI: 10.7554/eLife.32536

Detailed protocol

Growth, labeling and collection of 4TU samples

Day1

1. Inoculate 5mL of **half-ura media** and incubate at 30C overnight

Day2

1. Back dilute overnight culture in 100mL **half-ura media** to OD=.1, grow until at least OD = 0.45 at ≥ 150rpm at 30C (for wild-type in dextrose media, this typically takes about 4.5-5 hours)
2. Meanwhile - setup for collection:
   a. Filtration flask with enough filters (47mm diameter filter setup)
   b. Spatula + beaker with EtOH
   c. Liquid nitrogen
   d. Beaker of water to wash pipettes
   e. 10mL pipette
   f. Setup 1 cryo-tube per sample
3. Dilute this culture into 100mL of **half-ura media** at OD=0.45 and add 100uL of a 1M 4TU stock (1.0 mM final concentration, the stock is in DMSO and can be freeze/thawed 2-3 times) and start the clock
4. After 2 minutes (lag phase), collect the t=0 sample (8mL).
5. Process the t=0 sample by filtration, scraping the cells off the filter with the spatula and directly freezing the cell pellet in liquid nitrogen.
6. Continue to collect samples at the desired timepoints as described above (I typically take every 5 minutes for 55 minutes – 12 total timepoints)

Half-ura media:
800mL YNB
100mL 20% sugar
100mL 10x CSM -URA
5mL 100x uracil

Hot acid phenol RNA extraction and quantification

1. Fully resuspend cell pellet in 300uL TES buffer (10mM TrisHCl pH7.5, 10mM EDTA, 0.5% SDS) **+ 3ng spikes if spiking to culture volume (make a master mix of lysis buffer and spike to minimize pipetting errors, I use both a 4TU and non-4TU poly-adenylated RNA spikes)**
2. Add 300uL water or acid saturated phenol, incubate at 65C for 1 hour with constant vortexing
3. Incubate samples on ice for 5 minutes.
4. Spin samples at 4C for 10 minutes at max speed
5. Extract aqueous phase and add 300uL water/acid saturated phenol in a low-adhesion tube. Vortex and spin again at RT.
6. Extract the aqueous phase with 300uL chloroform in a normal tube.
7. Add 50uL 3M NaOAc pH5.2 and 400uL isopropanol in a low-adhesion tube. Incubate at -20 for at least 1 hour.
8. Pellet the RNA and wash once with 75% ethanol.
9. Resupend the cell pellet in 100uL RNase free water.
10. Quantify the RNA with the nanodrop. The concentration should be 800-1400 ng/uL.

Use low-adhesion tubes for all subsequent steps in this protocol!!
Scale up by a factor of two when preparing for deep sequencing experiment

Biotinylation with MTSEA-biotin

1. Dilute 14ug RNA into 105uL total volume with DEPC water **(+ 1ng spike RNA if spiking to total RNA (make a master-mix of the water and spike to minimize pipetting errors)**.
2. Add 15uL HE buffer.
3. Add 30uL 100ug/mL MTSEA-biotin in DMF.
4. Rotate in the dark for 60 min.
5. Add 150uL acid phenol:chloroform, vortex, let sit for 2 min and spin for 5 min.
6. Extract aqueous phase and add 17uL 5M NaCl and 200uL isopropanol
7. Precipitate and resuspend pellet in 14uL water.

HE buffer: 100mM HEPES pH 7.5 10mM EDTA

mRNA enrichment (this can be started when the streptavidin beads are blocking)

1. Set a heat block to 65C and another one to 80C. Chill **oligo-dT elution buffer** on ice.
2. Spin the biotinylated RNA sample at max speed for 2 minutes to pellet any residual biotin and add 12uL biotinylated RNAs (should be ~10-12ug) to 12uL **oligo-dT binding buffer**. Avoid any white material that has pelleted!
3. Heat the RNA mixture to 65C for 2 minutes, then place on ice immediately.
4. Add 24 uL oligo-dT bead slurry to a microfuge tube (Invitrogen Dynabeads Oligo$(dT)_{25}$) and remove supernatant using the magnet and a pipette (a vacuum line will be too powerful).
5. Wash the beads twice with 12uL **oligo-dT binding buffer**.
6. Resuspend beads in 12uL **oligo-dT binding buffer**.
7. Add RNA/binding buffer mixture to beads and rotate at room temperature for 5 minutes.
8. Remove supernatant with a pipette and discard. Wash the beads twice with 24uL **oligo-dT washing buffer**.
9. Resuspend beads in 20uL **oligo-dT elution buffer** and incubate at 80C for 2 minutes.
10. Place beads immediately on magnet and collect eluted mRNAs as quickly as possible and put into a new tube or if proceeding directly to streptavidin selection, into the tube with the streptavidin beads.

Oligo-dT binding buffer: 20mM TrisHCl pH7.5, 1M LiCl, 2mM EDTA
Oligo-dT washing buffer: 10mM Tris Hcl pH7.5, 150mM LiCl, 1mM EDTA
Oligo-dT elution buffer: 10mM TrisHCl pH7.5

Streptavidin selection

Washing and blocking the streptavidin beads

1. Thaw **50x Denhardt's reagent**.
2. Add 25uL streptavidin bead slurry to a microfuge tube (Invitrogen Dynabeads MyOne Streptavidin C1) and remove the supernatant using the magnet and a pipette (a vacuum line will be too powerful here).
3. Wash the beads twice with 25uL **strept Buffer 1**, once with 25uL **strept Buffer 2** and twice with 25uL **strept buffer 3**.
4. Resuspend the beads in 25uL **strept Buffer 3** and add 2.5uL **50x Denhardt's reagent** (5x final concentration).
5. Rotate beads for 20 minutes.
6. Wash beads four times with 75uL **strept Buffer 3**.
7. Resuspend beads in 25uL **strept Buffer 3** and add 2uL 5M NaCl.

Streptavidin subtraction

1. Setup the following:
    a. Set a heat block to 65C.
    b. 75uL **strept Buffer 3** heated to 65C per sample.
    c. 100uL 5% BME in water per sample.
    d. Thaw 5mg/ml linear acrylamide (Ambion)
2. Add the purified mRNAs to the blocked streptavidin beads and rotate for 15 minutes.
3. Collect flowthrough and save in a fresh tube.
4. Wash beads with 75 uL **strept Buffer 3** prewarmed to 65C, combine wash with flowthrough.
5. Wash beads with 75 uL **strept Buffer 4** and add this wash to the flowthrough.
6. Wash beads with 75 uL **strept Buffer 5** and add this wash to the flowthrough.
7. Wash beads again with 75 uL **strept Buffer 5** and add this wash to the flowthrough.
8. Add 25uL 5%BME to each tube, resuspend beads and incubate on a rotator for 5 minutes.
9. Collect this first eluate using the magnet.
10. Add another 25uL 5%BME to each tube, resuspend beads and incubate in a 65C block for 10 minutes.
11. Collect the second eluate and combine with the first. Add 3uL acrylamide, 7uL 5M NaCl and 150uL isopropanol and incubate at -20.
12. Put the combined flowthough and washes back on the magnet and let it sit for 5 minutes, transfer the liquid to a new tube to clean up any residual beads.
13. Add 25uL 5M NaCl, 3uL linear acrylamide and 400uL isopropanol to the flowthough and incubate at -20.
14. After at least 1 hr at -20, pellet the flowthrough and eluate samples by spinning at max speed for 10 minutes.
15. Wash the sample with 400uL 75% EtOH, remove the supernatant with a pipette, the pellet can be **very** slippery at this point.
16. Air dry in the hood for 5 minutes.
17. If processing for DNase treatment, resuspend the pellet in 18uL water + 2uL DNase buffer. The pellet will take 15-30 minutes to resuspend with occasional agitation. Then add 0.5uL DNase to each sample and incubate at 37C for 20min and remove the DNase with either the anti-DNase beads or extract the RNAs with acid phenol:chloroform (50/50).

50x Denhardt's Reagent: 1% polyvinylpyrrolidone, 1% Ficoll type 400, 1% BSA
Strept buffer 1: 0.1M NaOH
Strept buffer 2: 0.1M NaCl
Strept buffer 3: 1M NaCl, 10mM EDTA, 10mM TrisHCl pH7.4
Strept buffer 4: 10mM TrisHcl pH7.4, 1mM EDTA, 1% SDS
Strept buffer 5: 10% **Strept buffer 3**

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Chan, L. (2019). 4TU metabolic labeling and RNA analysis. Bio-protocol Preprint. bio-protocol.org/prep17.
2. Chan, L. Y., Mugler, C. F., Heinrich, S., Vallotton, P. and Weis, K.(2018). Non-invasive measurement of mRNA decay reveals translation initiation as the major determinant of mRNA stability. eLIFE. DOI: 10.7554/eLife.32536